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Molecules in focus

Antisense oligonucleotide therapies for Amyotrophic Lateral Sclerosis: Existing and emerging targets

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a disease with highly heterogenous causes, most of which remain unknown, a multitude of possible disease mechanisms, and no therapy currently available that can halt disease progression. However, recent advances in antisense oligonucleotides have made them a viable option for targeted therapeutics for patients. These molecules offer a method of targeting RNA that is highly specific, adaptable, and does not require viral delivery. Antisense oligonucleotides are therefore being developed for several genetic causes of ALS. Furthermore, biological pathways involved in the pathogenesis of disease also offer tantalizing targets for intervention using antisense oligonucleotides. Here we detail existing and potential targets for antisense oligonucleotides in ALS and briefly examine the requirements for these drugs to reach and be effective in clinic.

1. Introduction

Amyotrophic Lateral Sclerosis (ALS) has seemed until recently to be refractory to therapeutic intervention. However the advent of novel molecular medicines, in particular antisense oligonucleotides (ASOs), has brought with it the potential for treatment. There are currently around 35 genes linked to increased risk of ALS, accounting for approximately 15% of sporadic and 70% of familial cases (Renton et al., 2014). They collectively implicate diverse processes in ALS pathogenesis; RNA processing, protein quality control, and organelle and vesicle trafficking. Mechanistically, mutations in these genes act through gain-of-function, loss-of-function or both. Many of these genes also modify the behaviour or function of the RNA-binding protein TAR DNA binding protein 43 (TDP-43), making TDP-43 and its substrates therapeutically relevant targets.

Wildtype TDP-43 is deposited in protein aggregates in neurons and glia in ~96% of ALS cases, with aggregates in the remaining cases being composed of mutant TDP-43, mutant SOD1 or mutant FUS, decorated with degradation adaptor proteins including ubiquitin (Neumann et al., 2006; Vance et al., 2009). Macroaggregates of TDP-43 and other proteins are likely to be protective, but they signal the presence of pre-aggregates and oligomers which are increasingly predicted to be the toxic species (Scotter et al., 2014). Because oligomer formation is concentration-dependent, reducing protein concentration using targeted reduction therapies has the potential to prevent toxicity. Although the autosomal dominant inheritance patterns for the vast majority of ALS genes implicate gain-of-toxic-function mechanisms, loss of wildtype function is increasingly being reported. Respecting this balance is a major consideration for ASOs which target ALS-linked genes, and in this review we present the case that regulators of ALS genes or their downstream pathways may represent safer targets. In this review, we explore available and potential ASO therapies for this complex disease, examining the most promising targets, mechanisms of action, and modes of delivery to patients.

2. Biology and chemistry of antisense oligonucleotides

Distinct classes of therapeutic oligonucleotides exist; siRNAs, which engage with the RNA-induced silencing complex, and antisense oligonucleotides (ASOs), which do not. Both control gene expression post-transcriptionally through Watson-Crick base pairing, but they differ in their chemical composition, mechanisms of action, and site of predominant subcellular activity (cytoplasm for RNA interference and nucleus for ASOs) (Deleavey and Damha, 2012).

ASOs are short, synthetic, single-stranded molecules designed to complement a target RNA and either modify gene expression through enzymatic cleavage of the transcript, or sterically block the binding of proteins involved in RNA maturation or translation (Fig. 1). The oligo-

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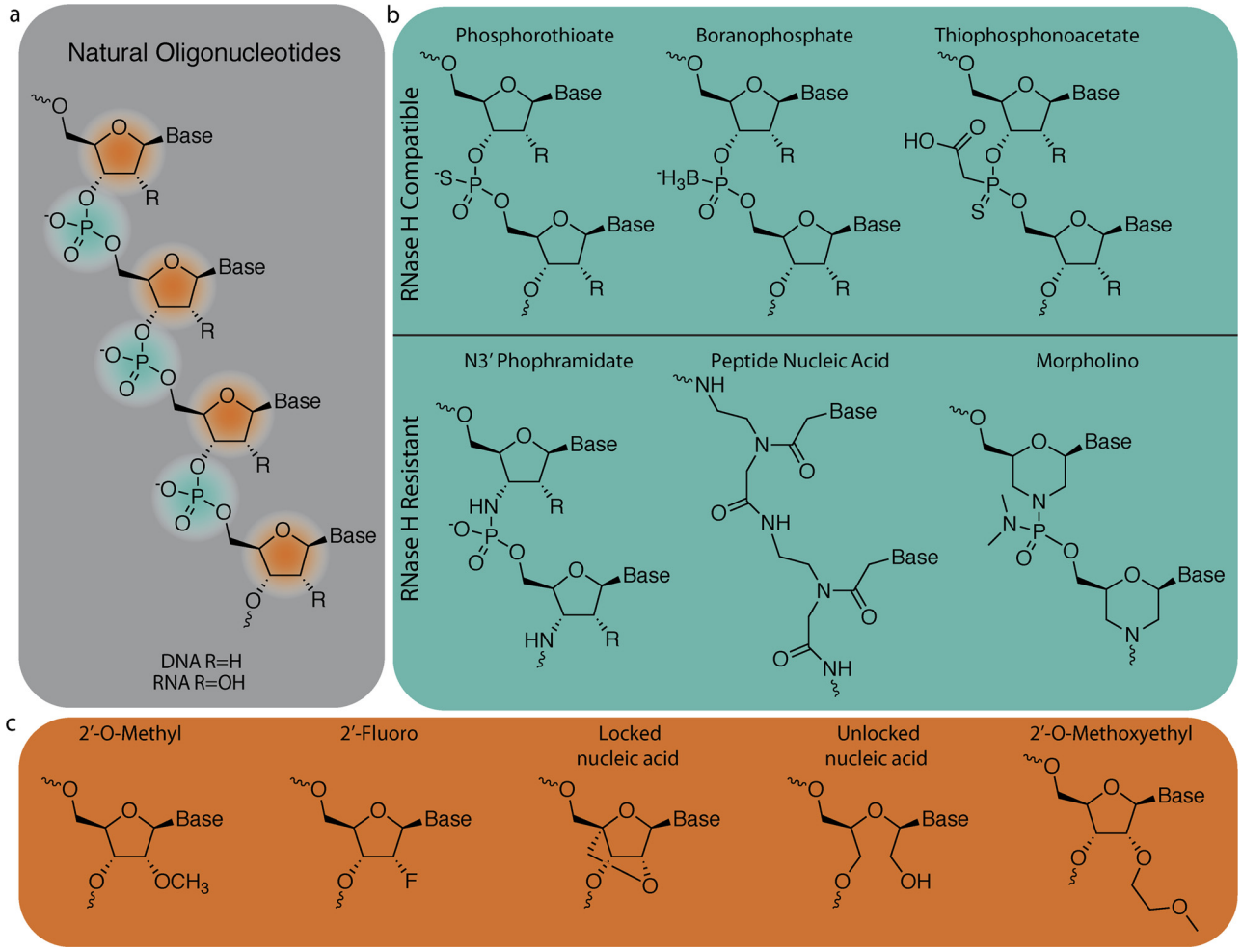

Fig. 1. Selected antisense oligonucleotide chemical modifications.
(a) The natural RNA/DNA structures are often modified at the internucleotide linkages (teal) and the sugar rings (orange) to improve nuclease resistance, increase target binding affinity, and enhance pharmacokinetics while reducing toxicity. (b) Modifications to the phosphodiester backbone can be employed to improve cellular uptake, make ASOs nuclease-resistant, and tune their ability to trigger RNase H cleavage. Gapmers contain a combination of chemical modifications. (c) Ribose is commonly modified at the 2' position to enhance target binding affinity, increase stability, and reduce immunostimulatory properties.

RNA duplexes formed can become substrates for the endonuclease RNase H, which results in cleavage of the target mRNA and potent silencing of the gene product (Lima et al., 2007). This approach, therefore, is ideal for forms of ALS caused by toxic gains-of-function of either the protein or RNA. Alternatively, chemical modifications to the ASO backbone can prevent RNase H recruitment, such that they instead modulate gene expression through steric blockade (Deleavey and Damha, 2012). Depending on the RNA target region, ASOs can prevent ribosome assembly and translation, displace translation inhibitory elements, alter binding of RNA stability modulators, or alter splicing and thus isoform expression. The splice-switching ASO SPINRAZA for spinal muscular atrophy displaces splicing factors to convert *SMN2* mRNA into *SMN1*-like mRNA, thus increasing protein expression of SMN1 (Finkel et al., 2017). Therefore, ASOs can either increase or decrease protein expression through distinct mechanisms making them a highly versatile therapeutic.

Until recently, the poor stability, low transfection efficiency, off-target gene silencing, and immunostimulation of ASOs hampered their clinical implementation. Chemical improvements to ASOs, including alternative phosphate linkages, backbone sugars, and nucleobases (Deleavey and Damha, 2012) (Fig. 1) have yielded modified oligonucleotides with increased binding affinity, nuclease resistance, cellular uptake and endosomal release, and reduced immune activation. Currently popular are 'gapmers' which use modified nucleotides flanking a central 'gap' of unmodified nucleotides capable of stimulating RNase H, including Ionis' first-in-human ALS therapy for *SOD1* (Miller et al., 2013). 'Stereopure' ASOs, such as Wave Life Sciences' WVE-3972-01 against expanded *C9ORF72* repeats, are individual ASOs with controlled chirality at each phosphorothioate linkage, selected from the racemic mixture of hundreds of thousands of isomers for their superior uptake and potency (Brown, 2017). Some ASO chemistries may be vulnerable to sequestration into stress granules by mutant RNA-binding proteins (Bailey et al., 2017), which are frequently found in ALS, and ASO-protein interactions can sometimes alter the potency of an ASO (Liang et al., 2015). Nevertheless, as human therapeutics for ALS, ASOs have many advantages over other nucleotide therapeutics including superior bioavailability, broad CNS distribution (with intrathecal injection), better cellular uptake and safety profile (Geary et al., 2015).

3. ASO targets in ALS

3.1. Direct targeting of mutant genes: allele-specific versus non-specific ASO targeting

For ALS cases where a genetic mutation is the clear cause, allele-specific ASO targeting is an attractive strategy because it offers the selective removal of the mutant but not the wildtype gene product. For TDP-43 and FUS there is clear evidence that the wildtype protein is

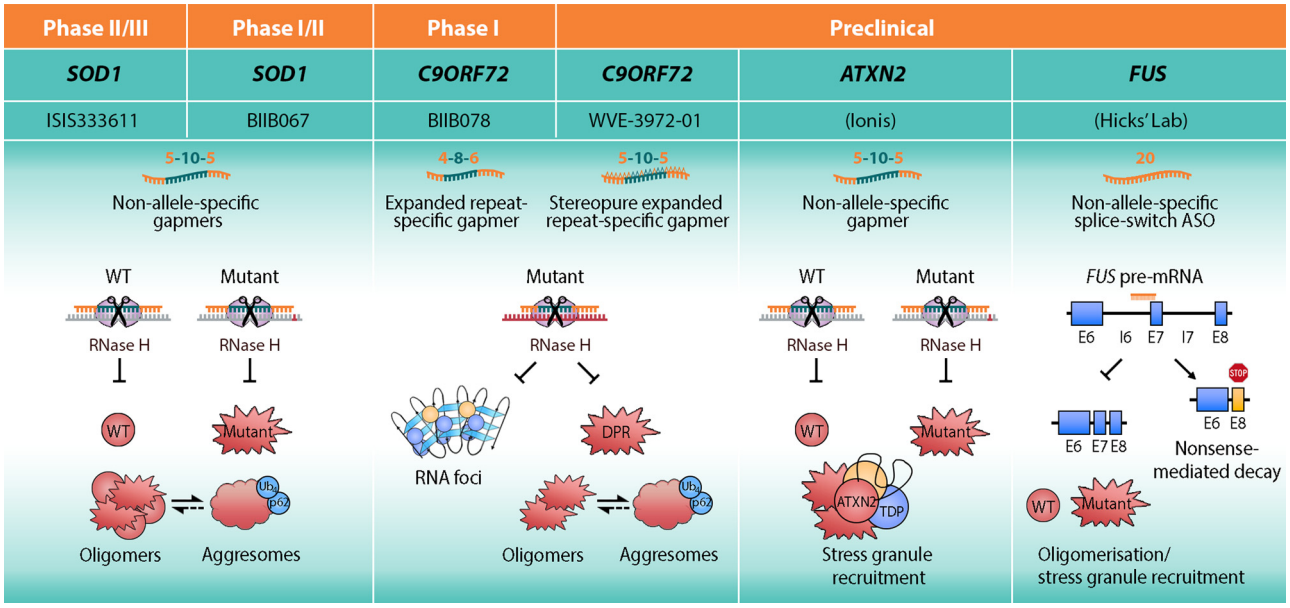

Fig. 2. Selected antisense oligonucleotide therapies in trial or development for ALS.
Three ASO-based therapies for ALS are currently in clinical trials with several more in development. *SOD1* ISIS333611, *SOD1* BIIB067, *C9ORF72* BIIB078, *C9ORF72* WVE-3972-01, and the *ATXN2* ASO are phosphorothioate (PS)- or mixed PS/phosphodiester-backbone gapmers with 2'-O-methoxyethyl-modified ribonucleotides flanking a central unmodified ribonucleotide stretch. For *C9ORF72* WVE-3972-01, the chirality of each phosphorothioate linkage is controlled. All five stimulate RNase H cleavage, of either the mutant transcript alone or of both wildtype and mutant transcripts, to mitigate gain-of-toxic-function; SOD1 or *C9ORF72* dipeptide repeat (DPR) polymers, *C9ORF72* RNA foci formation, or aberrant recruitment of TDP-43 to stress granules by ATXN2. The FUS splice-switching ASO has a PS-linked backbone with 2'-O-methyl-modified ribonucleotides, which targets the intron 6/ exon 7 junction to sterically block binding of auto-regulatory FUS protein and induce exon 7 skipping. The resulting frameshift produces a premature stop codon in exon 8, inducing nonsense-mediated decay of FUS transcript and decreased FUS protein.

essential for function; TDP-43 or FUS knockout from mouse embryos is lethal, and partial knockdown postnatally causes a progressive ALS-like disease (Iguchi et al., 2013; Hicks et al., 2000). Although *C9ORF72* gene knockout does not cause a motor phenotype in rodents, it is required for immune function therefore reduced expression of the wildtype gene could be detrimental (Sudria-Lopez et al., 2016). Even for *SOD1*, whose function may be dispensible for development (Reaume et al., 1996), chronic depletion in adulthood can induce motor neuron dysfunction (Fischer et al., 2011).

An allele-specific ASO for *C9ORF72,* Biogen's BIIB078, complements the expanded repeat to selectively target mutant transcripts for degradation, and is now in phase I trials (Fig. 2). Targeting upstream of the *C9ORF72* expanded repeat in animal models also selectively reduces repeat-containing transcripts, while ASOs targeted downstream reduce all isoforms (Lagier-Tourenne et al., 2013; Donnelly et al., 2013). An RNase-resistant ASO (full 2-O-methoxyethyl-modified) against the expanded repeat blocks RNA-binding protein recruitment to foci, which has been proposed as a pathomechanism (Donnelly et al., 2013). Because ASOs tend to target nuclear transcripts, the relative contribution to pathogenesis of RNA foci (nuclear) and dipeptide repeat-encoding transcripts (cytoplasmic) may determine whether *C9ORF72*-linked ALS can be treated by ASO therapy. In mutant *TARDBP* cases, allele-specific targeting may be possible, given that an allele-specific siRNA afforded near complete silencing of TDP-43^{M337V} expression in cell lines without affecting the wildtype protein (Nishimura et al., 2014). There are of course some foreseeable limitations to the use of allele-specific ASOs in ALS; i) even mutant-specific knockdown could risk loss-of-function effects (particularly for males with ALS caused by X-linked *UBQLN2* mutations), and ii) the need to develop and trial specific ASOs for individual mutations. We predict that 'personalised' mutation-specific ASOs will be among the next wave of therapies, but their feasibility will be dependent upon improved patient access to genotyping, a rapid development pipeline, and most importantly an expedited pathway to clinical approval. Alternatively, non-allele-specific ASOs may circumvent such issues.

A non-allele-targeting ASO for *SOD1* (ISIS333611) was the first ASO to be trialled in humans, reaching 21 patients with 15 different *SOD1* genotypes (Miller et al., 2013). A non-targeting ASO has also been developed for *FUS,* binding the intron 6/exon 7 junction to repress exon 7 inclusion and induce mRNA decay (Zhou et al., 2013) (Fig. 2). Although the debate is ongoing, the findings in the *SOD1* human trial of only partial SOD1 knockdown in the CNS, and the absence of peripheral SOD1 knockdown or loss-of-function toxicity, suggest that allele-specific *SOD1* ASOs to avoid loss-of-function toxicity are not yet an urgent need. Instead, the current challenge for ALS therapy researchers is to improve target knockdown in the broader patient base, using non-allele-specific ASOs.

3.2. *Upstream: regulators of RNA-binding proteins as ASO targets*

Given the risk of modifying certain ALS-causing genes directly, and the fact that the majority of ALS cases are sporadic, an emerging strategy is to use ASOs to target ALS-associated gene modifiers or pathogenic pathways. Pathological misfolding, mislocalisation or post-translational modification of wildtype TDP43 are features in almost all cases of ALS (Neumann et al., 2006), and these features are regulated by both the stress granule response and protein degradation pathways. Remarkable life extensions have been observed in neurodegenerative rodent models after reducing levels of the stress granule proteins ATXN2 (Fig. 2) and TIA1 (Apicco et al., 2018; Becker et al., 2017). The stress granule protein HNRNPA2/B1 is another intriguing potential target for knockdown; its mutation causes ALS but largely through gain-of-function mechanisms and it colocalizes to stress granules with TDP-43 (Martinez et al., 2016). Similarly, knockdown of endogenous inhibitors of protein degradation pathways (Lee et al., 2010) may a tractable strategy by which ASOs could accelerate the degradation of ALS-linked proteins. These being fundamental cellular processes, extensive preclinical testing of such ASOs will be necessary to rule out

toxicity. However, given that ASO-mediated reduction of *ATXN2* was safe and effective in mice, ASOs that modify TDP-43 through the stress granule response or other upstream targets offer the tantalising promise of a generic therapy for almost all cases of ALS.

3.3. Downstream: RNA-binding protein-regulated transcripts as ASO targets

Splicing aberrations are a major defect downstream of RNA-binding protein dysfunction (Colombrita et al., 2015; Humphrey et al., 2017). Both TDP-43 and FUS regulate many transcripts in a cell type-specific and species-specific manner, however several credible ALS-relevant targets have emerged including *POLDIP3*, *AGRN*, *ELAVL3*, and *MAPT*. Two recent studies identified a splicing defect in *STMN2*, a regulator of microtubule dynamics, motor neuron outgrowth, and regeneration that corresponded with downregulation of *STMN2* in human post mortem ALS tissue and which was downstream of TDP-43 dysfunction (Klim et al., 2019). Given the success of the splice-switching ASO SPINRAZA for spinal muscular atrophy (Finkel et al., 2017), a similar approach might be employed for misspliced targets of TDP-43 or FUS that contribute to neurodegeneration. Targets downstream of RNA-binding protein dysfunction might also include microRNAs. Human motor neurons expressing mutant FUS had decreased levels of the neuroprotective miR-375 (De Santis et al., 2017). Thus, methods to either activate or inhibit microRNAs downstream of ALS genes might also be considered.

4. Delivery of ASOs

The mode of ASO delivery to ALS patients is crucial. It is currently necessary to deliver ASOs via spinal (intrathecal) injection, in order that they by-pass the blood-brain barrier (BBB). While intrathecal administration minimises peripheral off-target effects, lumbar injections can be challenging, or cause adverse events including pain and headache (Miller et al., 2013). The risk of these events increases with regular injections, as is required for the spinal muscular atrophy ASO SPINRAZA which patients receive every 4 months (Finkel et al., 2017).

Two strategies could mitigate the requirement for repeated or intrathecal injection; extending ASO half-life and improving their permeability across the BBB, respectively, although achieving both may prove challenging. Phosphorothioate ASOs already benefit from low-affinity plasma protein binding that reduces their renal clearance, but their circulation lifetimes might be further extended by conjugation to lipid- or polymer-based 'nanocarriers' (Geary et al., 2015). However, many nanocarriers developed to date exhibit cytotoxicity, and are likely to further impede BBB transit (Juliano, 2016). ASO transport across the BBB may be best achieved by molecular-scale conjugation to substrates of endothelial uptake processes; ASOs coupled to anti-transferrin receptor antibody or to short positively-charged cell-penetrating peptides are transcytosed across endothelia via receptor-dependent or independent endocytosis (Juliano, 2016). Once they reach the CNS, cell type-specific targeting of ASOs may not be necessary given the likely role for non-neuronal cells in ALS pathogenesis (Neumann et al., 2006).

Another consideration for ASO delivery is duration, particularly with respect to the treatment of gene carriers in the presymptomatic phase. The risks to humans of prolonged ASO treatment will be difficult to examine preclinically, but once presymptomatic treatment is achievable, ASOs may bring us closer to disease prevention at least in familial cases.

5. Discussion

ASOs are inexpensive to manufacture and offer specific targeting of transcripts containing genetic lesions as well as upstream pathogenic events. Genes mutated in ALS with toxic gain-of-function mechanisms are clear targets for direct interference by antisense oligonucleotide therapies. However, a key question underpinning selective ASO therapies is whether the potential target causes toxicity through gain- or loss-of-function, or both. Ideally, an ASO should modify the levels or splicing of only mutant-bearing transcripts. However, sequence-specific differences in ASO safety profiles will need to be well understood before personalised ASOs become a clinical reality. Even then, considering that mutant alleles probably retain some wildtype function in addition to toxic gain-of-function, complete reduction of mutant-bearing transcripts may not be without consequence.

Furthermore, the majority of ALS cases are sporadic in nature with no 'mutant' target identified. Thus, an alternative route to employing oligonucleotide-based therapies will be to modulate pathways implicated in ALS disease pathogenesis, including protein homeostasis and stress granule assembly. These pathways are common not only to the vast majority of ALS but also to other neurodegenerative diseases, offering further economy of scale of synthesis, and power to measure efficacy. Sub-therapeutic targeting of several pathways could be another powerful way to mitigate the risks of long-term suppression of a single gene. Similarly, combining ASOs with currently available drugs or small-molecule TDP-43 modulators currently under investigation may allow for safer long-term therapy.

A number of challenges remain before the potential of ASO therapies for ALS can be realised. There is a clear trade-off between bringing ASO therapies to market quickly and delaying their availability in order to identify the most efficacious ASO design and chemistry. The use of preclinical model systems that accurately model disease should aid in screening for such ASOs and this might be iterative, whereby ASOs which have been tested in humans could become tools to identify preclinical models with the highest predictive value. The best ASOs are unlikely to afford disease reversal in humans, which would require regeneration of motor neurons. However, advancements in genetic diagnostics alongside the rapid development of ASO chemistries and targeting strategies mean that ASOs will be a key technology for halting ALS progression, which would represent a major advancement for this devastating disease.

Competing interests

None to declare.

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